ETV6-NTRK3 and *STRN-ALK* kinase fusions are recurrent events in papillary thyroid cancer of adult population

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Abstract

Objective: PTC-specific analysis identified novel fusions involving *RET*, *BRAF*, *NTRK1*, *NTRK3*, *AGK* and *ALK* genes in adults and pediatric PTCs. Although many novel fusions are PTC-specific events and, therefore, are ideal for diagnosis purposes, validation across additional and larger patient cohorts is essential for introducing these potential diagnostic or prognostic biomarkers into the clinical practice. As most of the *BRAF*, *NTRK3* and *ALK* fusions were initially found in pediatric PTC or in more aggressive thyroid carcinomas, and there is a great disparity across population, in this study, we screened a large set of adult-sporadic PTC cases for the most prevalent kinase fusion lately described in the TCGA.
Design and methods: The prevalence of the fusions was determined by RT-PCR in 71 classical PTC, 45 follicular variants of PTC (FVPTC), 19 follicular thyroid adenomas (FTAs) and 22 follicular thyroid carcinomas (FTCs).
Results: *ETV6–NTRK3* was exclusively found in FVPTC, in both encapsulated and infiltrative variants, but was not found in FTAs and FTCs. *STRN-ALK* was found in both classical PTC and FVPTC. No *AGK-BRAF* fusion was identified in this series, endorsing that *AGK–BRAF* is a genetic event mainly associated with pediatric PTCs.
Conclusions: The identification of kinase fusions in thyroid carcinomas helps to expand our knowledge about the landscape of oncogenic alterations in PTC. As *ETV6–NTRK3* and *STRN–ALK* are recurrent and not identified in benign lesions, they can certainly help with diagnosis of thyroid nodules. Further analysis is needed to define if they can also be useful for prognosis and guiding therapy.

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Introduction

Chromosomal rearrangements, which cause the production of a functional fusion gene, were the first mechanism of oncogene activation discovered in human cancers in 1973, particularly in hematologic malignancies (1, 2). The first gene fusion found in malignant epithelial tumors was reported in 1987, when the DNA isolated from a primary papillary thyroid carcinoma (PTC) was successfully delivered to NIH3T3 cells through transfection (3). Cytogenetic and molecular findings demonstrated that the gene fusion occurred due to a paracentric inversion of chromosome 10, which juxtaposes the intracellular tyrosine kinase domain of the *RET* to the 5′ sequence of the *CCDC6*, which is expressed in thyroid follicular cells (3, 4, 5). This novel fusion transcript was named *RET/PTC*, as it encompasses the *RET* gene and was exclusively found in PTC (3).

The following decade was characterized by the discovery of several novel fusions involving *RET* gene in both sporadic and radiation-induced PTCs. *RET/PTC* represents the major group of gene fusions found in PTC, with *RET/PTC1* and *RET/PTC3* being the most prevalent isoforms (reviewed in (6, 7, 8)). Importantly, *in vitro* and *in vivo* analyses confirmed that *RET/PTC* fusions have a critical role in thyroid carcinogenesis (9, 10, 11).

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Recently, advances in genomic technologies have uncovered novel fusion genes in thyroid tumors. The Cancer Genome Atlas (TCGA) Pan-Cancer project has generated comprehensive multidimensional maps of the key genomic changes in 33 tumors types, including PTC. PTC-specific analysis not only confirmed the significance of known fusions (*RET* and *NTRK1*) but also identified novel fusions involving kinases that very likely play a role in thyroid cancer such as *BRAF*, *NTRK3* and *ALK* (12).

Although thyroid cancer showed lower frequency of kinase fusions compared to other tumor types, a high percentage of these events were recurrent, confirming their role in the pathogenesis of thyroid carcinomas. Among the novel gene fusions, some isoforms involving *BRAF*, *NTRK3* and *ALK* genes were acknowledged to play a role in the pathogenesis of pediatric PTC or more aggressive variants of PTC.

Regarding fusions involving the *BRAF* gene, *AGK–BRAF* was primarily described in post-Chernobyl radiation-induced thyroid tumors (13) and later identified as a recurrent event in sporadic pediatric PTC (14). Moreover, among the isoforms identified, *AGK–BRAF* was functionally characterized and showed to be able to induce MAPK activation and to increase cell proliferation and transformation in NIH3T3 or COS7 transfected cells (13).

ETV6-NTRK3 is the most common rearrangement found after any *RET/PTC* isoform in the TCGA (12). While its prevalence was very low (1%) in PTC from adults (12, 15), it is the second most common rearrangement in radiation-exposed PTC (13, 15). Additionally, *ETV6-NTRK3* has been shown to be able to induce MAPK activation and increase cell proliferation and transformation (13). Although other novel isoforms of *NTRK3* fusions were described, only one sample was found to be positive for the *NTRK3* fusion and the transforming potentials of these fusion proteins were not determined.

Finally, concerning *ALK* fusions, *STRN–ALK* was found as a very rare event identified in more aggressive variants of thyroid cancer (16) and in conventional PTCs (17). Importantly, its ectopic expression was able to induce MAPK activation, increase cell proliferation and transformation and induce tumor formation in xenograft models (16). Although novel ALK fusion variants were described in PTC, the isoforms were not found to be recurrent or were not functionally characterized.

Unlike *RET/PTC*, these fusions were not found in adjacent normal thyroid tissues or benign neoplasms (12, 13, 16, 17), which support the pathogenic role of these fusions in PTC.

As genetic and environmental variations contribute to population-based disparities and we enter the era of precision medicine and molecular diagnosis, we questioned about the implications of these novel fusions in the diagnosis of PTC from different geographical areas. Additionally, validation analysis in a large and independent set of malignant and benign lesions is indubitably essential to better define the relevance of these novel fusions in the pathogenesis of thyroid tumors and, consequently, in the differential diagnosis of thyroid nodules.

We here reported the prevalence of *ETV6-NTRK3*, *STRN–ALK* and *AGK–BRAF* fusions in a large Brazilian cohort of adult-sporadic PTC. We additionally assessed the prevalence of these fusions in other benign and malignant thyroid tumors.

Methods

Sample selection

The series consists of primary tumors from patients who underwent thyroid surgery from 2000 to 2007 at Hospital São Paulo, Universidade Federal de São Paulo and Hospital das Clínicas, Faculdade de Medicina da Universidade de São Paulo. The study included 116 PTCs, 71 (61%) classical PTCs and 45 (39%) follicular variants of papillary thyroid carcinoma (FVPTC). The final histological classification on surgical specimens was obtained from formalin-fixed paraffin-embedded sections. The FVPTC is defined as a tumor composed entirely or almost entirely of cells with a follicular architecture exhibiting nuclear features of classical PTC. The FVPTC can be completely encapsulated (EFVPTC) or partially encapsulated or infiltrative (IFVPTC). Additionally, 19 follicular thyroid adenomas (FTAs) and 22 follicular thyroid carcinomas (FTCs) were used to investigate the presence of *ETV6–NTRK3*, *STRN–ALK* and *AGK–BRAF* in other benign and malignant thyroid tumor subtypes. None of the patients had a history of previous radiation exposure. Informed consent was obtained from all subjects involved in this study. The study was conducted under the approval of the Review Boards and Research Ethical Committees of the affiliated institutions. The demographic and clinico–pathological features such as the age at onset, gender, tumor size, histological variant, multifocality, extrathyroidal invasion, the presence of lymph node metastasis and recurrence were correlated with the genetic profile.

Detection of fusion transcripts by standard RT-PCR

All the samples were screened for the presence of *ETV6–NTRK3*, *STRN–ALK* and *AGK–BRAF* by standard RT-PCR. For the detection of fusion transcripts, primers were designed within exons located near the breakpoint regions described in PTC (Fig. 1 and Table 1).

Total RNA was isolated from the core of the tumor (in an attempt to avoid contamination with normal tissue) using Trizol (Invitrogen, Thermo Fisher Scientific), according to the manufacturer's instructions. Total RNA concentration and purity were analyzed using a Nanodrop ND-2000 spectrophotometer (Thermo Fisher Scientific). RNA integrity was evaluated using agarose gel electrophoresis or Agilent 2100 Bioanalyzer System (Agilent Technologies). Total RNA (1 µg) was treated with DNAse and reverse-transcribed into cDNA with oligo-$dT_{12–18}$ (0.5 µg) using a Superscript III reverse transcriptase kit (Invitrogen) as described (18). An aliquot of cDNA (1 µL) was subjected to PCR amplification using 10 mM Tris–HCl (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$, 200 µM of each dNTP, 1 unit of Platinum Taq DNA polymerase (Invitrogen) and 200 nM of each specific primer for the target genes or reference gene (*RPS8*). PCR reaction was performed as follows: initial denaturation at 95°C for 5 min followed by 40 cycles of 95°C for 30 s, annealing temperature for 30 s and 72°C for 30 s, followed by a final extension of 72°C for 2 min. The PCR products were analyzed by electrophoresis on a 2% agarose gel and visualized on a Bio-Rad Gel Doc EZ system (Bio-Rad Laboratories). The presence of the fusion transcripts was confirmed by direct sequencing of PCR products using the BigDye Terminator Cycle Sequencing Kit (Thermo Fisher Scientific) as previously described (19). The primers used to detect fusions, reference gene, annealing temperatures and expected PCR product sizes are detailed in Table 1.

Expression of fusion oncogenes in PCCL3 cell line

PCCL3 (normal follicular thyroid cells derived from Fischer rats) were cultured in Ham's F12 medium supplemented with 5% FBS (Gibco, Thermo Fisher Scientific) and 4 hormone-mixture including thyrotropin (1 U/mL), hydrocortisone (10 nM/mL), transferrin (5 µg/mL) and insulin (10 µg/mL) (Sigma-Aldrich). PCCL3 cells were transiently transfected with the expression vector pMSCV-*ETV6–NTRK3* or pLVX-AGK-BRAF-GFP, as previously

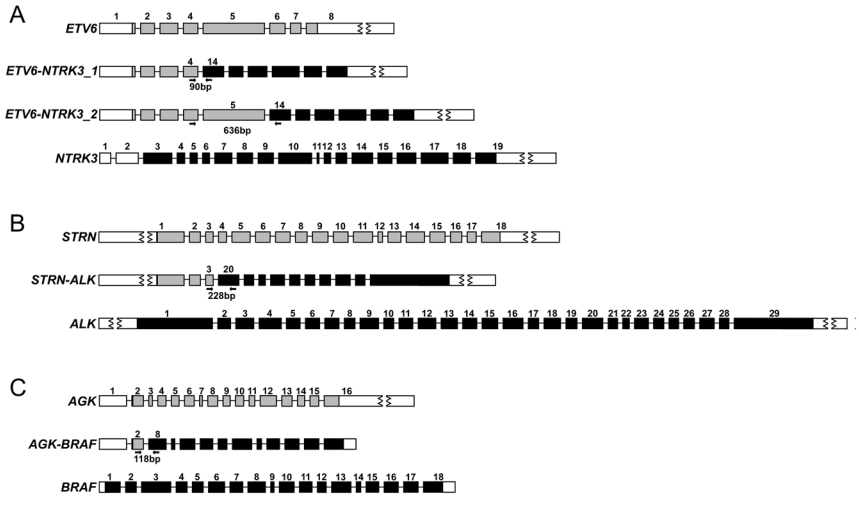

Figure 1

Diagram of exon/intron structure of *ETV6*, *NTRK3*, *STRN*, *ALK*, *AGK* and *BRAF* genes and the fusion transcripts described in thyroid carcinomas. (A) *ETV6* (gray; NM_001987) and *NTRK3* (black; NM_002530) genes and the fusion transcripts of *ETV6–NTRK3*. *ETV6–NTRK3_1* (Cosmic ID: COSF1535) and *ETV6–NTRK3_2* (Cosmic ID: COSF1537) isoforms correspond to the fusion of exon 4 or exon 5 of *ETV6* with exon 14 of *NTRK3*, respectively. (B) *STRN* (gray; NM_003162) and *ALK* (black; NM_004304) genes and the *STRN–ALK* fusion transcript (Cosmic ID: COSF1431). (C) *AGK* (gray; NM_018238) and *BRAF* (black; NM_004333) genes and the *AGK–-BRAF* fusion transcript. Full blocks (gray and black) represent the coding region, and empty blocks represent the untranslated region (UTR). The numbers refer to the exons. The arrows indicate the position of the primers used to amplify all fusion transcripts described in thyroid carcinomas.

Table 1 PCR primers, annealing temperatures and expected PCR product sizes.

Gene fusions	Primer sequence (5′–3′)	Temp (°C)	Location	Expected product size (bp)
ETV6–NTRK3[a]	F: ACACACACAGCCGGAGGTCATAC R: AGTGGGCTGGCTGAGTCCTCC	60	Exon 4 Exon 14	90/636[b]
STRN–ALK	F: GCAACCTTATCCGACTTCTAGC R: GATACTGGTGCCCGCTCTC	61	Exon 3 Exon 20	228
AGK–BRAF[a]	F: CTGCTGACCTGGGGAGGCCATT R: TCATCTGCTGGTCGGAAGGGCTG	60	Exon 2 Exon 8	118
RPS8	F: AACAAGAAATACCGTGCCC R: GTACGAACCAGCTCGTTATTAG	60	Exon 3 Exon 4	104

[a]Ricarte-Filho *et al*. (15); [b]product sizes for both *ETV6-NTRK3_1* (90 bp) and *ETV6-NTRK3_2* (636 bp).

described (20). *ETV6–NTRK3* and *AGK–BRAF* plasmids were kindly donated by Dr James Fagin (Memorial Sloan-Kettering Cancer Center). The oncogene-transfected cells were harvested, and the total RNA was isolated using TRIzol Reagents (Invitrogen) and reverse-transcribed into cDNA using oligo$(dT)_{12–18}$, as aforementioned. The cDNA generated from cells expressing the fusion transcripts was used as a positive control. For the *STRN–ALK* detection, an aliquot of cDNA from a PTC sample known to be positive for *STRN–ALK* was used as positive control.

Statistical analyses

The association between the tumor size and the type of genetic alteration was determined using one-way ANOVA test followed by Bonferroni's *post hoc* test. To determine the association between the type of genetic alteration and the clinical–pathological features, Fisher's exact test was used with the raw data, and the percentage was shown in the graphics. Statistical analyses were performed using GraphPad Prism v5.01 Software (GraphPad Software).

Results

ETV6–NTRK3 was exclusively found in follicular variants of PTC

As two isoforms of *ETV6–NTRK3* were previously identified in PTC (13), the set of primers used in this study were designed to detect both isoforms (Fig. 1 and Table 1). Overall, the *ETV6–NTRK3* fusion transcript was found in 5% ($n=6$) of PTC cases. All positive cases harbor an in-frame fusion of exon 4 of *ETV6* to exon 14 of *NTRK3* (Fig. 2A and Table 2). Remarkably, *ETV6–NTRK3* was exclusively found in FVPTC, leading to a prevalence of about 13% (6/45) in this variant (Table 3). Essentially, 50% were infiltrative follicular variants of papillary thyroid carcinoma (IFVPTC) and 50% were encapsulated follicular variants of papillary thyroid carcinoma (EFVPTC) (Table 3). One IFVPTC patient, a 23-year-old female, had lymph node metastasis tissue available and, therefore, it was also screened for the presence of *ETV6–NTRK3* transcript. The metastatic lymph node tested was also positive for the *ETV6–NTRK3* fusion transcript (Fig. 2B). Although non-invasive EFVPTC have very low risk of adverse outcome and were recently reclassified as non-invasive follicular thyroid neoplasia with papillary-like nuclear features (NIFTP), no additional tumor slides and blocks were available to further re-evaluate whether tumors are either NIFTP or invasive EFVPTC.

STRN–ALK was detected in about 3% of PTC samples

The *STRN–ALK* fusion transcript was found in about 3% ($n=4$) of PTC samples (Fig. 2C, Tables 2 and 3). All the fusions found were in accordance with the breakpoint already described, corresponding to the in-frame fusion of exon 3 of *STRN* with exon 20 of *ALK* (Fig. 2C). The fusion was found in both classical and follicular variants of PTC (Table 3).

No *AGK–BRAF* was detected in this series of PTC

Samples were also examined for *AGK–BRAF* fusion transcript. No PTC case was found to be positive for *AGK–BRAF* (data not shown), suggesting that *AGK–BRAF* fusion is mainly associated with pediatric PTC.

ETV6–NTRK3, *STRN–ALK* and *AGK–BRAF* are restricted to PTC

Since most of the fusions were found in FVPTC (78%; 7/9) and as its molecular profile seems to be closer to the FTA/FTC group, the prevalence of *ETV6–NTRK3*, *STRN–ALK* and *AGK–BRAF* was also investigated in a series of FTA ($n=22$) and FTC ($n=19$). The evaluated FTC and FTA

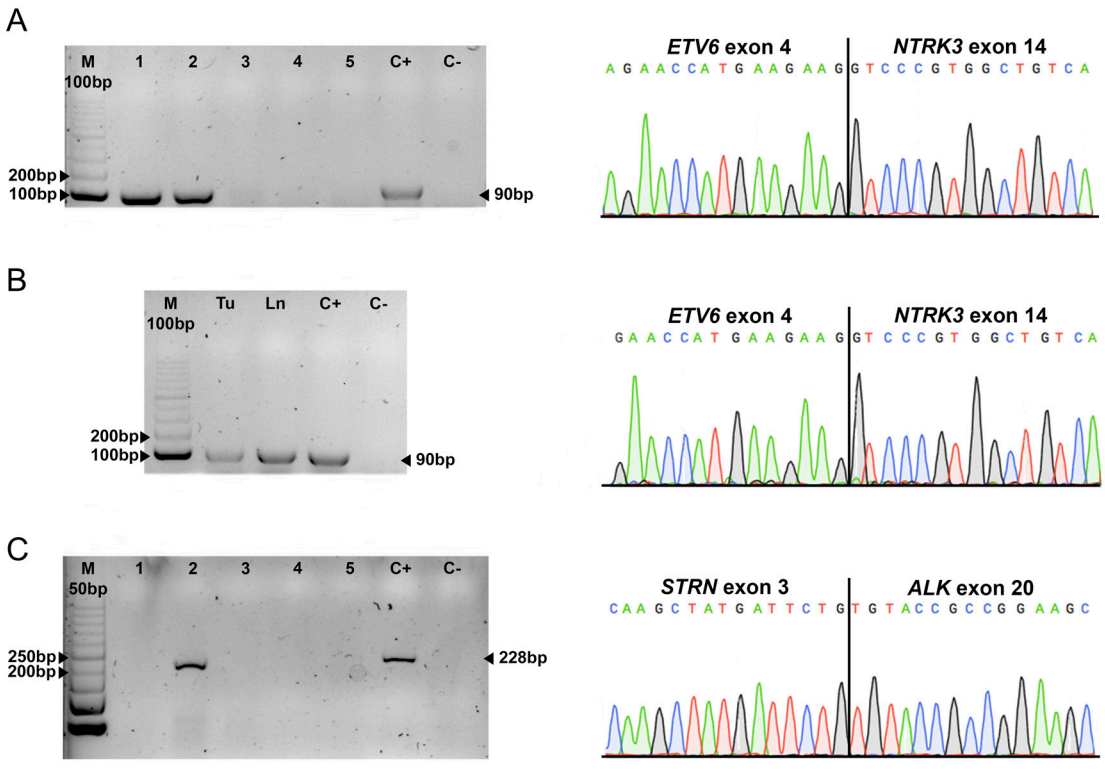

Figure 2

Fusion transcripts detected in sporadic PTCs. (A) *ETV6–NTRK3* fusion transcript identified in sporadic PTCs by standard RT-PCR. Positive (lanes 1 and 2) and negative (lanes 3, 4 and 5) PTC samples are shown. (B) *ETV6–NTRK3* fusion transcript in a primary metastatic tumor (Tu) and its matched lymph node metastasis (Ln). (C) *STRN–ALK* fusion transcript in a sample positive (lane 2) and sample negative (lanes 1, 3, 4 and 5). PCR products submitted to Sanger sequencing to confirm the presence of the fusions. Positive (C+) and negative controls (C–) were included in each run.

samples were negative for these 3 fusions, endorsing that they are specifically associated with PTC subtype.

Gene fusions are found in younger patients and are not associated with aggressive characteristics

When samples were divided based on the presence of *ETV6–NTRK3* or *STRN–ALK*, there was no statistically significant association between with clinico–pathological parameters and the presence of *NTRK3* or *ALK* fusions. When samples with *ETV6–NTRK3* and *STRN–ALK* fusions were pooled together with those samples who harbor RET/*PTC* fusions (21) and compared with samples harboring BRAF V600E (19) or NRAS Q61 (21) point mutations or samples that were tested negative for fusions and mutations, we found that fusions were more common in younger patients (<45 years old; mean age 36.8±10.0) than mutations (<45 years old; mean age: 41.7±12.3). The difference was statistically significant when compared to samples that bear neither mutations nor fusions (<45 years old; mean: 52.2±14.1; $P<0.01$) (Fig. 3A and B). Samples harboring point mutations have a more aggressive behavior than samples negative for mutations (Fig. 3C).

Discussion

The comprehensive analysis of the mutational landscape of PTC (12, 22) has provided insights into the molecular alterations that drive its carcinogenesis. Transcriptome sequencing has been particularly useful in helping to identify novel fusions. However, an important question concerns the clinical impact of the found fusions: Are these novel fusions recurrent across multiple patients, tumor subtypes and populations and, therefore, provide the chance to improve the preoperative diagnosis of thyroid nodules and risk stratification?

In this study, we found that the novel fusions *ETV6–NTRK3* and *STRN–ALK* (12) are recurrent in this series of PTC from a different geographic area, while they are not

Table 2 Prevalence of fusion in radiation-induced and sporadic PTC.

Reference	Samples	*ETV6–NTRK3*, *n* (%)	*STRN–ALK*, *n* (%)	*AGK–BRAF*, *n* (%)
Radiation-induced PTC				
(15)[a]	26	2 (8%)	NE	1 (4%)
(17)[b]	62	9 (14%)	NE	NE
(31)[c]	67	4 (6%)	NE	NE
Total	155	15/155 (10%)	NE	1/26 (4%)
Sporadic PTC				
Pediatric				
(15)[a]	27	2 (7%)	NE	0 (0%)
(16)[a]	30	NE	NE	3 (10%)
(32)[a]	17	3 (18%)	NE	NE
(33)[a]	28	5 (18%)	NE	NE
(28)[d]	9	0 (0%)	1 (11%)	0 (0%)
Adult				
(34)	20	1 (5%)	0 (0%)	0 (0%)
(17)	151	3 (2%)	NE	NE
(14)	256[e]	NE	3 (1%)	NE
(13)	29	NE	2 (7%)	NE
(12)	496	5 (1%)	1 (0.2%)	1 (0.2%)
(21)	125	6 (5%)	1 (1%)	0 (0%)
(28)[d]	32	1 (3%)	0	0
This study	116	6 (5%)	4 (3%)	0 (0%)
Adult and pediatric				
(27)	262	NE	2 (0.7%)[f]	NE
Total		32/1021 (3%)	14/1345 (1%)	4/855 (0.5%)

[a]Studies performed in samples of pediatric patients with <18 years; [b]study performed in patients who were aged <18 years at the time of Chernobyl accident; [c]study performed only with young adult patient samples (<22 years old); [d]in this study, only patients <40 years old ($n=41$) were further elucidated. The data shown in this table are only from these patients. No data about fusion partners from patients >40 years old ($n=303$) were available in the article; [e]in this study, samples were classified as differentiated thyroid carcinomas; [f]information about age was not available. *STRN–ALK* fusion was observed in one pediatric patient (13 years old) and in one adult patient (50 years old).
NE, not evaluated.

present in benign lesions and, therefore, may have an important impact on the diagnosis of thyroid nodules in adult population from different populations. *AGK-BRAF*, on the other hand, is not found in adult population and could have an important impact only in PTC from pediatric population. Whether they might have impact on the prognosis, it is still not clear.

Regarding *NTRK3* fusions, although several 5′ partners were detected in PTC, the majority of them were present in only one sample (12). Interestingly, the *ETV6–NTRK3*

Table 3 The demographic and clinico–pathological features of PTCs positive for *ETV6–NTRK3* and *STRN–ALK* fusions.

ID	Sex	Age (years)	Size (cm)	Histological variant	Multifocality	ETE	LNM	Recurrence
ETV6–NTRK3								
1[a]	F	43	2.5	IFVPTC	No	No	No	No
2	M	23	2.5	IFVPTC	Yes	Yes	Yes[b]	Yes
3	F	31	NA	EFVPTC	No	NA	No	No
4	F	48	2.8	EFVPTC	No	No	No	No
5	F	42	2.0	IFVPTC	Yes	Yes	No	NA
6	F	NA	3.0	EFVPTC	No	No	No	No
STRN–ALK								
1[a]	F	43	2.5	IFVPTC	No	No	No	No
7	F	43	3.5	FVPTC[c]	No	No	No	No
8	F	47	1.5	Classical	Yes	Yes	No	No
9	M	34	2.2	Classical	Yes	No	Yes	No

[a]Patient 1 was positive for both *ETV6–NTRK3* and *STRN–ALK*, and also for *RET/PTC2* (30); [b]lymph node metastasis from patient 2 was also positive for *ETV6–NTRK3*; [c]information regarding the presence/absence of capsule is not available.
ETE, extrathyroidal extension; LNM, lymph node metastasis; F, female; M, male; NA, not available; IFVPTC, infiltrative (non-encapsulated) follicular variant of papillary thyroid carcinoma; EFVPTC, encapsulated follicular variant of papillary thyroid carcinoma.

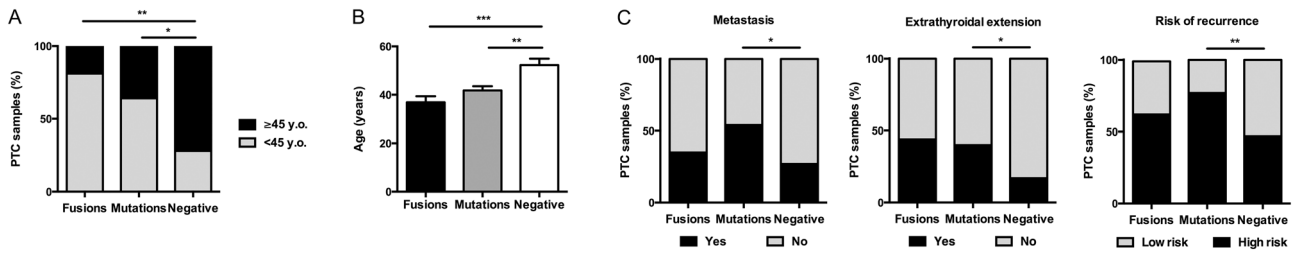

Figure 3

PTC samples divided into 3 groups according to the mutational status. Fusions: PTC samples with *ETV6–NTRK3*, *STRN–ALK* and *RET/PTC* (21) fusions. Mutations: PTC samples with point mutations in *BRAF* gene (19) or *NRAS* (21). Negative: those who bear neither fusions nor mutations. (A) Patients were classified according to age (≤45 vs >45 years old). Fusions were more prevalent in younger patients than negative. (B) Mean age of diagnosis of patients harboring fusions (age 36.8 ± 10.0), mutations (41.7 ± 12.3) or negative (mean: 52.2 ± 14.1). (C) Percentage of patients with the presence of metastasis, extrathyroidal extension and risk of recurrence in each group (fusion, mutation and negative). $*P<0.05$; $**P<0.01$.

fusion was identified as a common event in PTCs from patients who were exposed to radiation (15), and was also reported in sporadic pediatric (13) and adult PTCs (15). Afterward, it has been confirmed that *ETV6–NTRK3* is a recurrent event in PTC (12, 22) and is also able to induce MAPK activation and promote cell growth and transformation (13). Two *ETV6–NTRK3* isoforms have been identified in thyroid samples (15). Most of the fusions reported involve exon 4 of *ETV6* and exon 14 of *NTRK*3, but one case involved fusion of exon 5 of *ETV6* and exon 14 of *NTRK*3 (13). Therefore, we investigated the prevalence of *ETV6–NTRK3* fusion in a large set of sporadic PTCs, using a set of primers that would allow us to detect both isoforms. We here identified *ETV6–NTRK3* in six PTC cases. The prevalence (5%) is slightly higher than previously reported in adults but still lower than that reported in radiation-exposed PTC. Remarkably, all *ETV6–NTRK3*-positive cases were from FVPTC.

FVPTC is recognized as a tumor composed of follicles rather than papillae, but with cells presenting the nuclear features of PTC, and can be further divided into infiltrative (IFVPTC) or encapsulated (EFVPTC). It has been suggested that infiltrative tumors were more likely to have extrathyroidal extension and lymph node metastases, and its biological behaviors are similar to the conventional PTC, while the encapsulated behaves more like follicular thyroid tumors. At the molecular levels, it has been suggested that the non-invasive EFVPTC have a high prevalence of mutations that are usually associated with follicular-patterned thyroid tumors such as FTA and FTC (22, 23, 24). As we found *ETV6–NTRK3* fusion in three EFVPTC, we tested whether other follicular-patterned lesions, such as the benign FTAs and the malignant FTCs, could harbor *ETV6–NTRK3* fusion. None of the benign FTAs or malignant FTCs were positive for *ETV6–NTRK3*. Therefore, *ETV6–NTRK3* fusion has the potential to serve as a diagnostic marker for FVPTC. Since FVPTC represents a diagnostic challenge on fine-needle aspiration cytology and the percentage of follicular-patterned thyroid malignancies that proved to be FVPTC on final biopsy has increased, these findings have important clinical implications.

Regarding the prognostic implications, most *ETV6–NTRK3*-positive cases were under the age of 45 years, two cases had extrathyroidal extension and one case had lymph node metastasis. When both the paired primary tumor and the lymph node metastases were profiled, the *ETV6–NTRK3* fusion was also found in the lymph node metastases. The fact that this fusion was found in IFVPTC, as well as in the paired lymph node metastases, raises the possibility that tumors with *ETV6–NTRK3* fusion might have the potential to metastasize. However, whether additional mutations are needed to tumor progression, we still do not know.

Although *ALK* fusions are rare in thyroid cancer, aberrant ALK activation due to rearrangements leads to constitutive activation of the MAPK pathway in both PTC (16) and other tumor subtypes (25, 26). While originally found in PTC with more advanced stage of disease and tumors prone to dedifferentiation (16), *STKN–ALK* fusion has also been described in classical PTC (17, 22). Although other ALK fusion was described in medullary thyroid carcinoma (MTC) (27), *STKN–ALK* fusion was not found in FTC and MTC (16). In our cohort, four PTCs exhibited the *STRN–ALK* fusion. Although no association was found with demographic and clinico–pathological features such as histological subtypes, age and aggressiveness, one of the four PTC samples positive for *STRN–ALK* had lymph

node metastasis at diagnosis. Interestingly, the *STRN–ALK* fusions found in pediatric PTCs were metastatic or of a solid variant, indicating its association with a more aggressive phenotype even in pediatric samples (28, 29).

Importantly, the ALK inhibitor, crizotinib, has been shown to be effective in the treatment of patients with thyroid tumors harboring *STRN–ALK* (30) or other ALK fusions (27). A more detailed understanding of the downstream targets these fusions affect and the cellular process they are involved may help to find novel therapeutic targets.

Although common, a wide range of different 5′ partners were found to be fused in-frame to *BRAF*, and most were non-recurrent events (12). *AGK–BRAF* was found as a recurrent event in sporadic pediatric PTC samples (14) and in one radiation-exposed PTC case (13); however, in the TCGA cohort, only one PTC exhibited *AGK–BRAF* fusion. No *AGK–BRAF* fusion was found in over 100 adult-sporadic PTCs, endorsing that *AGK–BRAF* is an event likely associated with PTCs of younger age, while BRAF V600E is the leading genetic event in adult-sporadic PTC (12, 22).

Finally, nearly 26% (30/116) of all PTC samples in this cohort are negative for the most prevalent events described in PTC (19, 21).

The integrated view of *RET/PTC* fusions previously reported in this cohort (21), and those reported here, supports that these kinase fusions are recurrent events in PTC. Additionally, fusions were more prevalent in younger patients (<45 years old), while point mutations were associated with a more aggressive tumor phenotype. The identification of these fusions in thyroid carcinomas not only helps to expand our knowledge about the landscape of PTC but it also suggests that *ETV6–NTRK3* and *STRN–ALK* are useful for screening and therapeutic targets. Further analysis is still needed to delineate the comprehensive panorama of PTC.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this study.

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